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(54) Title: LANTHIONINE-CONTAINING ANTIMICROBIAL COMPOUND

(57) Abstract

A lanthionine-containing antimicrobial peptide obtainable from Staphylococcus hyicus strain 664 and pharmaceutically acceptable acid addition salts thereof as well as pharmaceutical compositions comprising said peptide or its acid addition salts and DNA molecules encoding said peptide are described. The peptide is purified from the culture medium of Staphylococcus hyicus strain 664 and can be used in a method of treating or preventing mastitis in a mammal comprising administering to said mammal a pharmaceutically effective amount of the peptide or a pharmaceutically acceptable acid addition salt thereof to effectively treat mastitis or effectively to suppress the rate, severity, and duration of subsequent bacterial infection.

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Lanthionine-containing antimicrobial compound

The present invention generally relates to a lanthionine-containing antimicrobial peptide obtainable from the culture medium of the bacterial strain Staphylococcus hyicus strain 664, to a pharmaceutically acceptable acid addition salt thereof, and to a method of producing said peptide. The invention further relates to a pharmaceutical composition comprising the lanthionine-containing antimicrobial peptide according to the invention, and to a method of producing said composition. The lanthionine-containing peptide according to the invention or its acid addition salts can be used as a pharmaceutical agent, but especially as a therapeutic agent in the treatment or prevention of bovine mastitis (anti-mastitis drug). Teat dipping drugs are included..

A large number of antimicrobial peptides exhibiting a broad activity spectrum is described in the literature. Amongst these the lanthionine-containing peptides represent an interesting group of antimicrobials because they exhibit a broad spectrum of activity against bacterial pathogens. Typical representatives are subtilin, cinamycin, nisin, duramycin, ancovenin, Ro 09-0198, pep5, gallidermin and epidermin. Some of these peptides are highly active against many bacteria including those causing bovine mastitis. Nisin for example is already used as a preservative in the food industry and in cosmetic preparations. Peptide antimicrobial agents have the advantage that they are digested by the consumer and this may make them non-toxic to warm-blooded animals. Accordingly, some of these peptide antimicrobial compounds have already been proposed for use in mastitis treatment, and any residues remaining in the milk should pose no risk to human health.

Mastitis is an inflammatory disease of the mammalian mammary gland. In veterinary medicine the most important and the most frequently encountered mastitis is that of dairy cows. Dairy cattle are highly specialised for milk production. They produce much more milk than is needed to nourish a calf. This super production, and the convention of milking dairy cows 2 or at most 3 times during 24 hours, renders their mammary glands susceptible to bacterial infections. In addition, they are milked by a mechanical apparatus that passes from cow to cow and so infection is transmitted from one animal to another.

The mammary gland has a number of natural defense mechanisms against bacterial pathogens (Cullor et al., 1990). These can be overcome by high levels of bacterial challenge and also by compromise of the defense mechanisms. This compromise can be brought about by poor management or through physiological changes at certain times in the

lactation cycle (Cullor et al., 1990). The period around drying off and calving are associated with a relatively high incidence of mastitis.

Mastitis can be caused by many species of bacteria. Those most commonly implicated in bovine mastitis fall into 2 categories I and II: Category I embraces host pathogens such as Staphylococcus aureus and Streptococcus agalactiae. These live on the skin of the udder or in the udder and individual cows are the source of infection to others in the herd. Category II embraces environmental pathogens such as Streptococcus uberis and Escherichia coli. As their name suggests these category II bacteria are found in the immediate environment of the dairy cow and thus present a constant risk (Cullor et al., 1990).

Mastitis caused by the bacteria characterized above can manifest as either clinical or subclinical disease (Cullor et al., 1990). Clinical disease can vary from mildly affected quarters with changes in the milk through severely infected quarters with eventual loss of that quarter, to a systemically ill cow that may die. Milder presentations are more usual.

Subclinical mastitis as its name suggests is not obviously present. It is, however, very prevalent in many dairy herds. Subclinically affected quarters have bacteria present and the cell content of milk is greater than normal. This syndrome is accompanied by lower production. Indeed, it has been estimated that up to 70 % of the economic losses sustained by farmers because of mastitis can be attributed to lost production from subclinical disease (Philpot, W.N., 1984).

Currently mastitis is controlled through the exercise of scrupulous hygiene at milking, by detection of chronic subclinically infected cows and either milking them after the

non infected cows or even by eliminating them from the herd. Clinical cases are generally treated with antibiotics as they occur. This means that milk must be withheld from sale and consequently causes economic loss to the farmer. Antimicrobial therapy of subclinical mastitis must be done in the dry period. Consequently, during the period between establishment of infection and drying off the infected cow is a threat to her neighbors.

It is, therefore, an object of the invention to provide an antimicrobial compound that may be advantageously used in the treatment or prevention of mastitis, especially in cows, and more especially in cows, which are not in the dry period.

This object was surprisingly met within the scope of the present invention by providing a further lanthionine-containing antimicrobial peptide or a pharmaceutically acceptable acid addition salt thereof that may be used as a pharmaceutical agent, but proved especially useful in the treatment of bovine mastitis. The lanthionine-containing antimicriobial peptide according to the invention which is also designated hyicin M51 is obtainable from the culture medium of the bacterial strain Staphylococcus hyicus strain664 using for example consecutive steps of conventional column chromatography. It has a molecular weight of about 2119 ± 2 Dalton and can be cleaved by trypsin to yield two fragments of a molecular weight of about 1256 ± 2 Dalton and about 881 ± 2 Dalton, respectively.

The present invention is therefore drawn to a lanthionine-containing antimicrobial peptide obtainable from the culture medium of the bacterial strain Staphylococcus hyicus strain 664, or a pharmaceutically acceptable acid addition salt thereof; to an antimicrobial or pharmaceutical composition comprising said peptide in addition to a suitable carrier, and to methods for the preparation of said peptide and said compositions. The invention further comprises the use of the inventive peptide and compositions to treat or prevent mastitis in a mammal or to prepare a drug for treating or preventing mastitis in a mammal. Finally the invention contemplates a method of treating or preventing mastitis in a mammal comprising administering to said mammal a pharamaceutically effective amount of the lanthionine-containing antimicrobial peptide according to the invention or a pharmaceutically acceptable acid addition salt thereof.

In a preferred embodiment of the invention the antimicrobial peptide according to the invention is prepared by

(a) growing a culture of the bacterial strain Staphylococcus hyicus strain 664 in a medium and for a time period appropriate to produce said peptide,
(b) purifying the peptide from the culture medium applying conventional techniques of protein purification, and
(c) drying the active fractions.

The bacteria are heat-inactivated in a first step by exposing the culture to temperatures between 50° C and 80° C, but preferably between 60° C to 70° C, for a time sufficient to heat-inactivate the bacteria. In the case of, for example, 65° C a period of 30 to 40 minutes of exposure will be sufficient. After heat-inactivation the bacteria are removed from the culture medium using filtration or centrifugation and the cleared supernatant is applied to a

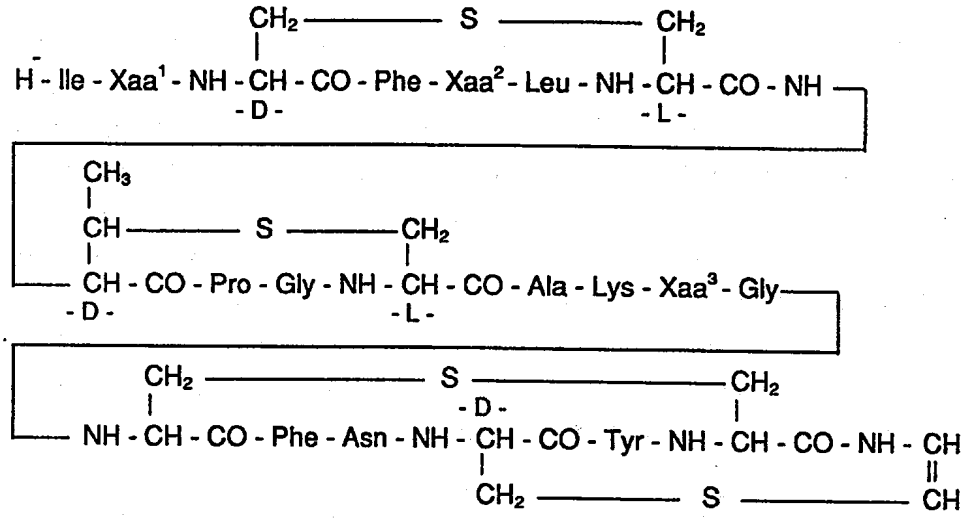
first chromatographic column. Alternatively, the column material can be added directly to the bacterial culture without prior removal of the bacteria. After a time sufficient to allow adsorption of the peptide to the chromatographic material, the resin is filtered off, washed and packed into a column. In a preferred embodiment of the invention Amberlite XAD-7 (Sigma) is used, in which case stirring of the culture in the presence of the resin for 1 to 3 hours ensures efficient adsorption of the peptide to the resin, which is washed after filtration with water and a solution of Na-citrate containing 20% to 30% isopropanol. After package of the resin into a column the peptide is eluted from the resin using for example a solution of Na-citrate containing 40% to 50% isopropanol in the case of an Amberlite XAD-7 resin. Active fractions are pooled and further purified in a similiar fashion using chromatographic materials such as for example SP Sepharose Fast Flow (Pharmacia). For storage purposes it is preferred to pool and lyophylise the fractions containing the antimicrobial peptide.

If the peptide is to be used for analytical purposes it can be further purified using for example HPLC. In a preferred embodiment of the invention RP-HPLC is done on a Vydac C18 column monitoring the protein by UV detection. In the case of RP-HPLC on a Vydac C18 column the peptide according to the invention can be eluted from the column applying a linear gradient of acetonitrile in 0.1% TFA, elution being followed by drying of the active fractions applying vaccuum centrifugation.

Purified hyicin M51 shows a strong antimicrobial activity against bacteria, especially against bacteria commonly implicated in mammalian and preferably bovine mastitis. Such bacteria include but are not limited to Staphylococcus aureus V557, Staphylococcus aureus Newbould 305, Streptococcus uberis, Streptococcus faecalis, Pasteurella haemolytica, Listeria innocua, Bacillus cereus, Micrococcus luteus, and Leuconostoc mesenteroides, and the Minimal Inhibitory Concentrations (MICs) for these bacteria are in the range from 0.01 g/ml to 100 g/ml, but preferably from 0.05 g/ml to 50 g/ml, and most preferably from 0.1 g/ml to 10 g/ml.

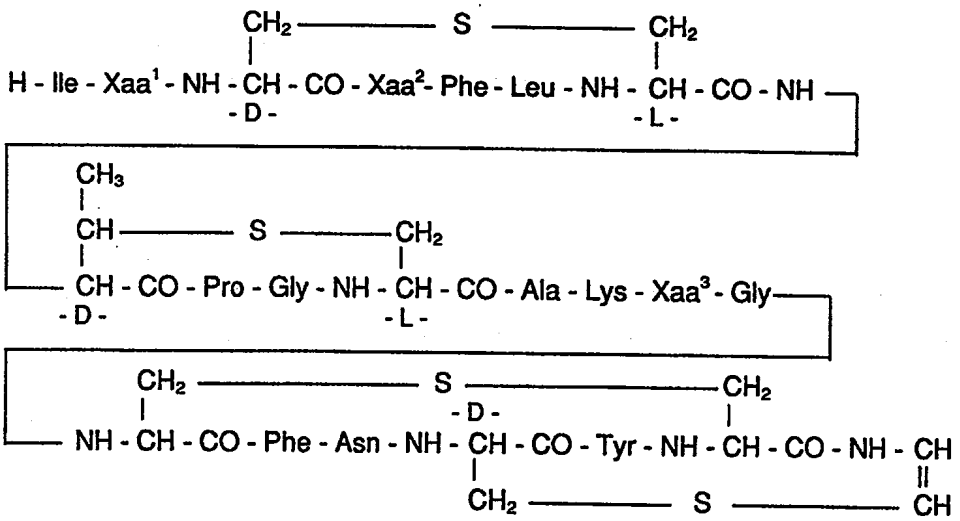
Amino acid sequence analysis of hyicin M51 is complicated by the fact that the second amino acid after the N-terminal Ile is an unusual amino acid blocking further sequence analysis from the N-terminal end. Therefore, the amino acid sequence has to be determined by the combination of experimental data obtained from mass spectroscopy, NMR spectroscopy, and DNA sequencing. The sequence as determined is highly homologous to the lanthionine-containing peptide gallidermin (EP-342486-A1), which has a molecular

weight of about 2164 ± 2 Dalton. The following peptide structure of hyicin M51 is revealed by amino acid sequence analysis



wherein Xaa^1, Xaa^2, and Xaa^3 can be any amino acid with the proviso, that the molecular wheight of the complete peptide is 2119 ± 2 Dalton. In a preferred embodiment of the invention Xaa^1 and Xaa^3 are 2,3-Didehydrobutyrin (Dhb), and Xaa^2 is 2,3-Didehydroalanine (Dha).

An alternative structure for hyicin M51 is



wherein Xaa^1, Xaa^2, and Xaa^3 can be any amino acid with the exception that Xaa^1, Xaa^2, and Xaa^3 must not be Ala, Lys, and α,β-Didehydrobutyric acid, respectively, within the same peptide.

The present invention also relates to an antimicrobial composition comprising in addition to a suitable carrier the lanthionine-containing antimicrobial peptide according to the invention in an antimicrobially effective amount. More specifically, the present invention relates to a pharmaceutical composition comprising in addition to a suitable carrier a pharmaceutically effective amount of the lanthionine-containing antimicrobial peptide according to the invention or of a pharmaceutically acceptable acid addition salt thereof. In a preferred embodiment of the invention a pharmaceutical composition is provided for the treatment and prevention of mastitis in a mammal, which preferably is a cow, comprising in additon to a suitable carrier a pharmaceutically effective amount of the antimicrobial compound according to the invention or one of the pharmaceutically acceptable acid salts thereof.

Within Staphylococcus hyicus strain 664 the prepeptide form of the lanthionine-containing antimicrobial peptide according to the invention is encoded by nucleic acid molecules. The structural gene of the prepeptide is encoded by bacterial DNA, whereas the template for translation of the prepeptide consists of RNA. Therefore, nucleic acid molecules encoding the lanthionine-containing antimicrobial peptide or the corresponding prepeptide constitute another embodiment of the invention. Preferably, the nucleic acid molecule comprises the nucleotide sequence

ATG GAA AAA ATT CTT GAT TTA GAT GTA CAA GTA AAG CGC AAA AAG GAA TCA
AAT GAT TCA TCT GGA GAT GAA CGT ATT ACA AGT TTC TCA TTG TGT ACT CCT
GGT TGC GCT AAA ACT GGT AGC TTC AAT AGT TAT TGT TGT TAA

or the nucleotide sequence

AUG GAA AAA AUU CUU GAU UUA GAU GUA CAA GUA AAG CGC AAA AAG GAA UCA
AAU GAU UCA UCU GGA GAU GAA CGU AUU ACA AGU UUC UCA UUG UGU ACU CCU
GGU UGC GCU AAA ACU GGU AGC UUC AAU AGU UAU UGU UGU UAA.

The nucleic acid molecules according to the invention can be obtained by a method comprising

(a) construction of a genomic library of Staphylococcus hyicus strain 664 in a microorganism;
(b) screening of said libary for sequences homologous to the structural gene of gallidermin;
(c) identification of a clone comprising the structural gene of hyicin M51; and
(d) purification of the nucleic acid molecule from the clone identified.

For the construction of said genomic library chromosomal DNA of Staphylococcus hyicus strain 664 is either partially or completely digested with a restriction enzyme. In the case of complete restriction digestion a suitable restriction enzyme preferably does not cut within the coding region of the hyicin M51 gene. The restriction fragments obtained are ligated to a plasmid vector either immediately after restriction digestion or after an optional size fractionation step. The resultant collection of various DNA fragments cloned within plasmid vectors is subsequently transformed into a microorganism. Preferably the microorganism used is E.coli or a bacteriophage infecting E.coli.

The genomic library obtained can be used to screen for sequences homologous to the structural gene of gallidermin. Screening procedures using hybridization with a suitable nucleic acid probe are described in Maniatis et al. Suitable probes can be obtained by restriction digestion of the gallidermin gene or by amplification of defined regions of the gallidermin gene using the polymerase chain reaction. Instead of radioactively labeled nucleotides, non-radioactive labels such as digoxigenin (DIG) labeled nucleotides can be used for labelling of the probe.

10^2-10^6 and preferably 10^3-10^5 clones of the genomic library are screened with the gallidermin probe. Positive clones, i.e. clones showing hybridization with the probe used, are further characterized by purifying and sequencing of the cloned DNA comprised by said clone, to identify the structural gene of the hyicin M51 peptide.

The structural gene of hyicin M51 can be used biotechnologically to produce hyicin M51 in a microbial host. For this purpose said host has to be equipped with the enzymatic machinery which performs the posttranslational modifications found in hyicin M51. It has been shown for several lantibiotics that a number of genes are involved in their production. The exact role of the corresponding gene products is not known in all cases, but there is evidence that they are required for processing of the pre-forms of the proteins, for the introduction of the modifications typically found in lantibiotics, as well as the translocation of the protein across

the membrane, and for immunity. In all cases studied so far these genes were found to be clustered around the structural gene for the lantibiotic. A comprehensive overview on the subject including the gene organisation of the epidermin operon can be found in "Nisin and Novel Lantibiotics" (Jung, G. and Sahl, H-G., 1991).

Since epidermin and hyicin M51 are structurally very similar it is reasonable to assume that the genes required for the production of hyicin M51 are also organized in an operon close to the structural gene as shown for lantibiotics. The DNA sequence of the structural gene for hyicin M51 constitutes a preferred embodiment of the present invention. Flanking sequences can easily be obtained by complete sequence analysis of the 6.5kb DNA fragment isolated from the genomic library of Staphylococcus hyicus which fragment comprises the structrual gene for hyicin M51. Additional clones comprising Staphylococcus hyicus genomic DNA adjacent to said 6.5kb fragment and clones of the whole operon can be obtained easily by procedures well known in the state of the art. A DNA molecule comprising the isolated operon can then be cloned in a plasmid which is able to be replicated and expressed in bacterial strains such as other strains of gram positive bacteria and staphylococci. Augustin et al (1991) for example describe the cloning and transfer of the epidermin genes from S. epidermidis to S. carnosus. Transfer of the genes necessary for the production of lantibiotics can also be achieved without detailed knowledge of all the genes involved. McKay et al (1984) and EP-137869 for example describe the transfer of nisin production by conjugative transfer. Hansen et al (1991) achieved transfer of subtilin production between two strains ob B. subtilis by integrative transformation.

Thus, microbial hosts devoid of the enzymatic machinery to produce hyicin M51 might be genetically modified in such a way as to be able to perform the posttranslational modifications needed. This could be achieved by expressing the genes encoding the enzymes responsible for the posttranslational modifications in said microbial host. The relevant genes might be introduced into the microbial host by way of transformation or conjugative transfer.

The application of antibiotics for treating mastitis commonly involves a severe problem, namely that of cheese and yoghurt manufacture. These processes are based on bacterial fermentation of milk and therefore residual agents with antimicrobial properties risk upsetting them.

It has also been established (Jung et al., 1992), that there is a significant reduction in the antimicrobial effect of these agents in the presence of milk, which causes reduced effectivity of mastitis treatment in cows with these agents.

It was therefore a long felt need to find an antibiotic peptide such as the peptide according to the invention which on the one hand fully controls bovine mastitis and is non-toxic to warm-blooded animals, and which on the other hand exerts only minor effects on bacteria in milk that play a role in cheese and yoghurt production.

Another embodiment of the present invention is directed to a method of treating or preventing mastitis in a mammal comprising administering to said mammal, which preferably is a cow, goat, or ewe, a pharmaceutically effective amount of the lanthinone-containing antimicrobial peptide according to the invention or one of the pharmaceutically acceptable acid addition salts thereof. The administration can be done either before or after the infection by the mastitis causing pathogens has occurred, and it can be done prepartum or postpartum.

As indicated, the present invention is concerned with treating or preventing mastitis. By "treating" is meant curing or ameliorating an animal that has contracted mastitis. "Preventing" mastitis means preventing the occurrence of the infection, or tempering the severity of the infection if it is later contracted.

It has surprisingly been found that the lanthionine-containing antimicrobial peptide according to the invention and pharmaceutically acceptable acid addition salts thereof are highly active against pathogens causing bovine mastitis, and what is even more surprising, they show almost no inhibition in their antipathogenic action in the presence of milk. What is most important, this lack of inhibition in the presence of milk allows reduction of the therapeutic dose and thus the amount of residual lanthionine-containing peptide in the milk such that the utilization of the milk from treated cows in cheese and yoghurt production is significantly improved.

Thus, one important aspect of the present invention is the treatment or prevention of mastitis in mammals by the administration of a lanthionine-containing antimicrobial peptide according to the invention or a pharmaceutically acceptable acid addition salt thereof to the subject mammal. A preferred embodiment of the invention is the treatment or prevention of mastitis in cattle. For this purpose the present invention contemplates employing any form

of the inventive peptides or pharmaceutically acceptable acid addition salts thereof alone or in combination. The present invention encompasses using native forms of the active agents. However, since the production of recombinant peptides has substantial advantages relative to the purification of native peptides, recombinant peptides are a preferred embodiment. It is also contemplated that synthetic forms of the antimicrobial peptides according to the invention and muteins showing minor structural differences to the native product exhibiting the same biological activity, are also within the scope of the invention.

The active agents of the present invention are usually prepared and stored as ready-to-use liquid formulations. The aqueous solution is generally applicable, but the formulation can also be adapted to the specific type of administration. Thus the formulation can also contain non-ionic surfactants that carry no discrete charge when dissolved in aqueous media and are selected from ethoxylated esters of fatty acids and triglycerides. The hyicin M51 formulation may also contain EDTA to improve the antimicrobial spectrum and stabilizing agents such as methionines, ascorbic acid, and preservatives such as propylene glycol.

Typically, the active agents of the present invention are administered by intramammary injection; however, effective dosages may be administered parenterally, percutaneously, by implant and also by dipping. In a preferred embodiment of the present invention the administration is carried out via intramuscular, subcutaneous, or intravenous injection. When prepared as injectables, the active agents according to the present invention are generally administered using a pharmaceutically acceptable vehicle or excipient. Suitable vehicles are, for example, water, saline, mannitol, dextran, amino acids, glycerol, or the like, in various combinations. In addition, if desired, the vehicle may contain auxiliary substances such as wetting or emulsifying agents, preservatives and pH buffering agents. The active ingredient will typically range from about 0.001% to about 95% (w/w) of the composition administered, or even higher or lower if appropriate.

Parenteral administration may be conventionally accomplished by subcutaneous, intradermal, intramuscular, and even intravenous injection. Needle-less air-blast injection devices may be equally useful. Parenteral administration is well known in the art and may be carried out in ways usual in the animal veterinary or human medical art.

Sustained action of the active agent to achieve prolonged release (so called 'slow release') can be obtained by formulating the protein in a matrix that will physically inhibit rapid dissolution. The formulated matrix is injected into the animal's body where it remains as a

depot from which the protein is slowly released. Useful adjuvants are polymers and copolymers of lactides and glycosides. Furthermore, gelling agents like aluminum, calcium or magnesium monostearate, or carbohydrates (cellulose, pectin, dextran derivatives), polysiloxanes or proteins (gelatin, collagen) could extend the releasing time of the active agents of the present invention after parenteral application. Percutaneous administration is also meant to include implantation of controlled release devices, e.g. made from silicone or wax, and other implantable matrices from polymeric materials can be used subcutaneously to deliver the compound over the required period of time. This can also be achieved by implantation of minipumps containing aqueous solutions of the protein. Such implantation techniques are also well known in the art and often used in medical treatment.

Polysiloxane carriers are described in the art for a variety of hormonal delivery forms and may be adapted to the release of the active agents of the present invention. A collagen delivery system for the release of antibiotics is described in the German Offenlegungsschrift DE-3,429,038. This system can also be adapted for the lanthionine-containing anitmicrobial peptides according to the invention and pharmaceutically acceptable acid addition salts thereof.

Slow release formulations and other pharmaceutical or veterinary formulations of the inventive peptide and its pharmaceutically acceptable acid addition salts can be prepared by adapting, for example, the formulation of lanthionine-containing peptides or other protein formulations already described in the art.

A "therapeutically effective amount" of an active agent of the present invention is a dose sufficient to either prevent or treat mastitis in a subject to which the active agent is administered. The dosages of the active agents of the present invention which can treat or prevent mastitis can be easily determined in view of this disclosure by one of ordinary skill in the art by running routine trials with appropriate controls. Comparison of the appropriate treatment groups to the controls will indicate whether a particular dosage is effective in preventing or treating a disease used in a controlled challenge. In general, effective dosage will vary depending on the mode of administration. It has been found that in the case of an intramammary injection using hyicin M51 administration of 0.1mg to 10mg per quarter is sufficient to control mastitis due to Staphylococcus aureus.

If administered intramuscularly, subcutaneously, or intravenously, effective dosages will depend on the weight of the animal and will typically run in the range of from about 2 g/kg

to about 800 g/kg, preferably 20 g/kg to about 200 g/kg. More typically, the dosage will be at least about 50 g/kg, but less than 150 g/kg.

Beyond dosage, an effective administration of an active agent according to the present invention will in part depend on the number and timing of the dosages. For example, multiple administrations of a dosage may be given to an animal, typically at least about 12hours apart. In most circumstances it may be desirable to administer the active agent at least three times. It may even be desirable to administer even more dosages to the animal, such as six, seven, eight, or even nine or more over an equal number of days or longer. It is believed that the precise combination of dosage and timing will be subject to a wide range of variation and that numerous combinations effective in treating or preventing a disease can be readily established by those of ordinary skill in the art in view of the present disclosure.

Reference will now be made to specific examples which are incorporated into the description for illustrative purposes and are not of a limiting nature unless there is a specific indication to the contrary.

EXPERIMENTAL

A) Preparation of Hyicin M51

The bacteriocin producer Staphylococcus hyicus 664 was obtained from the Czechoslovak National Collection of Type Cultures (CNCTC) and a sample of Staphylococcus hyicus strain 664 has been deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig on November 17, 1994, under the accession number DSM 9547.

An ISF 200 fermenter (Infors AG, Bottmingen, CH) containing 4 liters of BHI medium (Difco) with 0.05% antifoam Polywax Polyol PPG 2025 (BP Chemicals) was inoculated at 10% with an overnight culture of S. hyicus grown in BHI medium. The culture was grown at 37° C and 0.5bar backpressure. pO_2 was maintained at >60% of saturation by varying the aeration rate between 5-7lt/min and stirrer speed between 200-400rpm. (Antibacterial activity was detected by spotting aliquots of the samples on agar plates seeded with an indicator

bacteria as described in example F). After 6 hours the culture was heated to 65° C for 35 minutes to inactivate the bacteria. After cooling to 37° C the pH was adjusted to 4,9 with 3M HCL.

150g of Amberlite XAD-7 (Sigma) was added directly to the fermenter and stirring was continued for 2hours at 100rpm and an aeration rate of 7lt/min. The resin was filtered off and washed with 500ml of H20. It was then washed with 250ml of Na-citrate (20mM, pH4.0) followed by two washes of 250ml with the same buffer containing 20% isopropanol and two washes with this buffer containing 30% isopropanol.

The resin was then transferred to a chromatographic column (Pharmacia XK 50) and washed with another 1000ml of 20mM Na-citrate pH4 containing 30% of isopropanol. Activity was eluted with 20mM Na-citrate pH4 containing 30% of isopropanol, active fractions were pooled (750ml), and 11ml of SP Sepharose Fast Flow (Pharmacia) that had been prewashed with water were added to the pool. After stirring for 30min at RT the resin was filtered off, resuspended in 0.01M HCl and packed in a Pharmacia XK2.6 column. The column was washed with 100ml of 0.01M HCl containing 30% methanol. The activity was eluted with the same buffer containing 0.3M NaCl. Active fractions were pooled (120ml) and 11g of Amberlite XAD-7 (prewashed with H20) were added to the pool. After stirring for 45min the resin was filtered off and packed in a Pharmacia XK2.6 column. The column was washed with 100ml of 0.01M HCl and eluted with 0.01M HCl containing 50% isopropanol. The fractions containing activity were pooled and lyophilysed.

For analytical purposes the material was further purified by RP-HPLC on a Vydac C18 column (4.6mmx250mm). Activity was eluted using a linear gradient running from 75% solvent A, 25% solvent B to 60%A, 40%B. Solvent A was 90% H2O, 10% acetonitrile, 0.1% TFA, solvent B was 10% H2O, 90% acetonitrile, 0.1% TFA. Protein was monitored by UV detection at 213nm. Typically, fractions of 1ml were collected and analysed for activity. Active fractions were dried by vacuum centrifugation.

B) <u>Sequence analysis and amino acid profile of Hyicin M51</u>

Amino acid sequence information is obtained with a gas phase sequencer (Applied Biosystems, Foster City, California) using protocols supplied by the manufacturer.

The results show that after the N-teminal Ile the peptide is no longer available for sequencing indicating the presence of an unusual amino acid at position 2 of the peptide chain.

C) Mass Spectroscopy of Hyicin M51 and its tryptic fragments

Mass spectra are recorded on a Bio Ion 20252CF plasma desorption time of flight mass spectrometer (BIO ION KB, Uppsala, Sweden). The instrument is operated at an accelerating voltage of 15kV. A 1ns time resolution is employed.

2 g of sample are dissolved in 10 l of water - acidic acid (1:1 v/v) and added on an aluminium foil covered with a thin film of nitrocellulose. Nonadsorbed protein is washed off with water.

An enzymatic cleavage of the peptide with trypsin (Sigma, St. Louis, Mo) is carried out in a 0.1M bicarbonate buffer, pH8.5 for 15hours. The enzyme to substrate ratio is 1:10. Tryptic fragments are purified by RP-HPLC prior to mass spectroscopy. Results are presented in Table 1. A strong relationship to the structure of Gallidermin - another lanthionin containing bacteriocin - is noted.

Table 1: Mass Spectroscopy of Hyicin M51
Hyicin M51 and the two fragments TF1 and TF2 obtained by trypsin digestion are subjected to mass spectroscopy as described in example III.

Peptide	Molecular mass (M/Z)
hyicin M51	2119 2 Da
TF1	1256 2 Da
TF2	881 2 Da

D) **NMR Spectroscopy of Hyicin M51**

a) Experimental details

NMR measurements are done on a Varian Unity 500 spectrometer with a ^{1}H-frequency of 500MHz. CD_3OH is used as solvent. ^{1}H-NMR spectra are recorded at temperatures of between 17.5° C and 40° C with presaturation to suppress water. Two-dimensional spectra are recorded at 25° C, at which temperature the signal overlap in the amide region is minimal. For calibration purposes the CD_3-multiplett signal of the solvent CD_3OH is set to 3.30ppm.

In addition to one-dimensional ^{1}H-NMR spectra, the following measurements are performed: one-dimensional ^{13}C-NMR as well as two-dimensional DQF-H,H-COSY; TOCSY; NOESY; ROESY; H,C-COSY (HSQC); and H,C-COSY-long-range (HMBC)

b) Identification of individual amino acid residues

The identification of individual amino acids constitutes the first step in the elucidation of the peptide structure of hyicin M51 using NMR spectroscopy. ^{1}H-NMR signals obtained from one- and two-dimensional NMR experiments are identified as proton spin-patterns, which can be assigned to specific amino-acid residues. DQF H,H-COSY experiments provide proton signals, which are due to coupling over a distance of 2 or 3 chemical bonds; TOCSY experiments additionally provide proton signals, due to coupling over a distance of more than 3 chemical bonds; ROESY spectra provide proton signals resulting from dipolar coupling and provide additional information for the identification of individual spin systems; H,C-COSY experiments, finally, correlate protons, which are joined to a carbon atom directly.

The following natural amino acid residues are identified in hyicin M51:
Ile, Leu, 2x Gly, Ala, Pro, and Lys

The following non-natural amino acid residues of hyicin M51 are identified by their H,H-coupling patterns and chemical shifts:
2x Dhb (2,3-Didehydrobutyrin), Dha (2,3-Didehydroalanine), Abu (aminobutyric acid as part of 3-methyllanthionine), and Avi (2-aminovinyl)

Using ROESY and H,C-COSY spectra the following additional amino acid residues are identified:

Asn, 2x Phe, Tyr, and 6x Ala as part of lanthionine, 3-methyllanthionine, and 2-amino-vinylcystein.

The chemical shifts of the protons are summerized in Table 2.

c) Amino acid sequence (primary structure)

The sequential assignment of the individual amino acid residues is based on the interpretation of short-range ROESY cross signals between NH-, alpha- and beta protons of the i-th amino acid and the NH protons of the neighbouring amino acid i+1.

From the interpretation of the data obtained the following primary structure of hyicin M51 is established:

Ile Dhb Ala Phe Dha Leu Ala Abu Pro Gly Ala
Ala Lys Dhb Gly Ala Phe Asn Ala Tyr Ala Avi

d) Assignment of the thio-ether bridges of lanthionine,methyl-lanthionine, and 2-amino-vinyl-cystein

The characterization of the thio-ether bridges constitutes the last step in the elucidation of the structure of hyicin M51. Assignment of the various Ala structures to the different thio-ether bridges is achieved by interpretation of the long-range ROESY signals. The following thio-ether bridges are identified:

* 2 lanthionines between amino acid positions 3 and 7 and 16 and 21
* Methyl-lanthionine between amino acid positions 8 and 11
* A thio-ether bridge including 2-aminovinyl between amino acid positions 19 and 21

E) Cloning of the structural gene for Hyicin M51

a) Construction of a genomic library of Staphylococcus hyicus 664

Chromosomal DNA is isolated from a culture of S. hyicus 664 grown overnight at 37° C in M17-G medium (Terzaghi et al, 1975). Cells are harvested by centrifugation and lysed by incubation for 30 minutes at 37° C in 25mM Tris-HCl pH8, 10mM EDTA, 50mM glucose containing 4mg/ml lysozyme and 40mg/l mutanolysin.

Table 2: ^{1}H-NMR values in ppm of Hyicin M51 (CD_3OH, 10mmol, 25 C, TOCSY)

Aminosäure	NH	alpha	beta	gamma	delta	others		
Ile-1		3.92	2.02	1.66 1.30	0.98	1.10 (beta-Me)		
Dhb-2	fehlt		6.35	1.8				
Ala(Lan1)-3	7.79	4.55	3.08 3.04					
Phe-4	8.32	4.41	3.27 3.15			7.21 (ortho-H)	7.2-7.4 (meta-H)	7.2-7.4 (para-H)
Dha-5	fehlt		5.77 5.49					
Leu-6	8.46	4.35	1.77	1.70	0.96 0.91			
Ala(Lan1)-7	8.09	4.56	3.06					
Abu-8 (MeLan)	7.96	4.62	3.33	1.10				
Pro-9		4.35	2.38 1.80	2.05 1.80	3.32			
Gly-10	8.48	4.35 3.55						
Ala(MeLan)-11	8.00	3.81	3.58 3.01					
Ala-12	8.36	4.15	1.38					
Lys-13	7.77	4.41	1.91 1.81	1.50 1.47	1.70	2.96 (epsilon)		
Dhb-14	8.99		6.44	1.66				
Gly-15	7.68	4.21 4.00						
Ala(Lan2)-16	8.60	4.33	3.12 2.67					
Phe-17	9.24	4.92	3.55 2.81			7.13 (ortho-H)	7.2-7.4 (meta-H)	7.2-7.4 (para-H)
Asn-18	7.91	4.72	2.83 2.62			7.36 gamma-NH1	6.79 gamma-NH2	
Ala(CysAvi)-19	7.86	4.99	3.23 3.07					
Tyr-20	9.13	4.27	3.15 2.93			7.03 (ortho-H)	6.65 (meta-H)	
Ala(Lan2)-21	9.22	4.08	2.91 1.79					
Avi	8.43	7.01	5.67					

Dha ... 2,3-didehydroalanine
Dhb ... 2,3-didehydrobutyrin
Abu ... aminobutyric acid part of 3-methyllanthionine
Ala(MeLan) ... alanine part of 3-methyllanthionine
Ala(Lan) ... alanine part of lanthionine
Ala(CysAvi) ... alanine part of 2-aminovinylcystein
Avi ... 2-aminovinyl

Subsequently proteinaseK (100mg/l) and SDS (0.5%) are added to the sample which is then incubated for 2 hours at 56° C. After extraction with 1 volume of phenol/chloroform (1volume phenol:1volume chloroform) the DNA is purified by CsCl gradient centrifugation. The chromosomal DNA obtained is partially digested with the restriction enzyme Sau3A and the fragments obtained are size fractionated on sucrose gradients as described in Maniatis et al, 1982. DNA fragments of between 5kbp-10kbp are collected and ligated to BamHI linearized plasmid pUC18 which has additionally been treated with calf intestine phosphatase (CIP). The ligation mixture containing the ligation products is used to transform E.coli strain TG1 to ampicillin resistance.

b) Screening of the genomic library for sequences encoding Hyicin M51

The following two oligonucleotides complementary to the sequence of the structural gene encoding gallidermin (Schnell et al, 1989) are synthesized according to standard techniques:

OL1: 5'-CTTTTTGATCTTGACGTTAAAGT-3' (SEQ ID NO 2)

OL2: 5'-CCAATTTAATTCTTTTGAATT-3' (SEQ ID NO 3)

The oligonucleotides are used in a polymerase chain reaction (PCR) to amplify a 151bp DNA fragment of the gallidermin gene. For the polymerase chain reaction thermostable Taq polymerase from Perkin Elmer Cetus (GeneAmp™) is used according to the manufacturer's instructions. Genomic DNA of a strain of Staphylococcus saprophyticus, which is known to produce gallidermin, is used as the template DNA for amplification. To be able to use the amplified fragment as a probe for hybridization, the polymerase chain reaction is carried out in the presence of DIG-11-UTP purchased from Boehringer Mannheim.

About 10000 colonies of the genomic library obtained in section a) are screened with the DIG-labeled amplification product. The hybridization and washing steps are done as recommended by the manufacturer of the DIG-11-UTP nucleotides. Several clones, hydridizing with the DIG-labeled probe are identified. One of the clones is further characterized. It consists of pUC18 containing a 6.5kbp DNA insert. After cleavage of the plasmid with the restriction enzyme XbaI and subsequent religation, the size of the insert is reduced to about 1kbp. The sequence of this insert is determined using the Sequenase™ Version 2 Kit purchased from United States Biochemicals (USB) and appropriate oligonucleotides. The DNA sequence reveals an open reading frame of

144bp (SEQ ID NO 1) with considerable homology to the gallidermin structural gene gdmA. The deduced amino acid sequence (SEQ ID NO 4) establishes the results obtained from NMR analysis of the hyicin M51 peptide. After posttranslational modification mature hyicin M51 differs from gallidermin in three amino acid residues. The leader peptides of the two peptides show much less homology.

F) MIC plate assay

The MICs (Minimal Inhibitory Concentrations) of nisin and hyicin M51 against Streptococcus aureus and Streptococcus diacetylactis were determined. S. aureus Newbould 305 is a causative agent of Mastitis, S. diacetylactis is used in the manufacture of yoghurt and cheese.

Appropriate dilutions of nisin and hyicin M51 (300, 100, 30, 10, 3, 1, 0.3, 0.1 g/ml) were prepared in milk and in M17 broth (Merck) and allowed to stand at room temperature for 30 min.

Then, 10 ml aliquots of the solutions were spotted on M17 agar plates containing 0.5% of glucose. These plates had been overlaid with softagar (0.5% agarose) seeded with 1% of an A_{600}=1 culture of either S. aureus or S. diacetylactis. The spots were allowed to dry, then the plates were incubated at 30° C overnight.

The MICs were determined as the lowest concentration of antimicrobial protein causing the formation of a clear halo in the bacterial lawn. The results presented in Table 3 show that nisin and hyicin M51 have comparable activities against Streptococcus diacetylactis, both in milk and broth. There is however a clear inhibition of nisin activity against S. aureus in milk, while hyicin M51 retains full activity under these conditions.

Table 3: MIC plate assay (MICs for Nisin and Hyicin M51 in g/ml)

		milk	M17
Nisin	S. aureus	100	3-10
	S. diacetyl	1-3	1-3
Hyicin M51	S. aureus	3	10
	S. diacetyl	10	10

G) Effect of Nisin and Hyicin M51 on Staphylococcus aureus in milk

Staphylococcus aureus Newbould 305 was grown in M17 broth (Merck) at 37° C to an optical density at 600nm of 1.0. Cells were collected by centrifugation and washed in 20 mM Tris-HCl pH 8.0. The bacterial pellet was resuspended in milk at a density of about 10^7 cells/ml.

1 ml aliquots of this suspension were incubated in eppendorf tubes with various amounts of nisin and hyicin M51. Incubations were at 37° C for 30 min. A control with no antimicrobial added was run in parallel.

After incubation the samples were centrifuged to obtain the cellular pellet, which was washed 2 times with 1ml of 20mM Tris-HCl pH8.0 and resuspended in 1ml of this buffer. 100 l of the appropriate dilutions were plated on M17 agar plates containing 0.5% glucose and incubated overnight at 37° C. Colony forming units (cfu) were determined and percent survival was calculated relative to the control.

The results are shown in Table 4. It is clear from these data that in milk hyicin M51 has a much higher specific activity against Staphylococcus aureus than nisin.

Table 4: Effect of Nisin and Hyicin M51 on Staphylococcus aureus in milk

	% survival	
conc µg/ml	nisin	hyicin M51
100	0.2	
30	1.8	
10	15	0.01
3	65	1
1		12
0.3		29
control	100	100

It is well documented that nisin is inhibited in the presence of milk (Jung et al., 1992). Clearly and surprisingly hyicin M51 does not show a similar inhibition in the presence of milk. It is this particular property that suits it so well for the use as a prophylactic and

therapeutic agent for use in bovine mastitis since the therapeutic dose can be lowered accordingly.

To evaluate the potential of hyicin M51 or of its pharmaceutically acceptable acid addition salts in subclinical bovine mastitis caused by Staphylococcus aureus the infection model described below can be used.

H) **Antimicrobial spectrum of Hyicin M51**

The minimal inhibitory concentrations (MIC) of hyicin M51 against a panel of test organisms were determined using an agar well diffusion method. Agar plates were overlaid with soft agar seeded with the test organism. When the agar had solified wells of 4mm diameter were plugged into the agar. 50 l of a series of two fold dilutions of the peptide in citrate buffer pH4 were applied to the wells and the plates were kept at 4° C for two hours prior to incubation at 37° C overnight. The MIC was determined as the lowest concentration of peptide giving a clear zone of lysis around the well. Indicator strains were grown in Tryptose broth (Difco). M17 medium (Merck) was used for plates and soft agar except for Pasteurella haemolytica (Tryptose supplemented with 5% of fetal calf's serum), Leuconostoc mesenteroides (Plate count agar - Merck -, + 15% saccharose), and Bacillus cereus (Plate count agar + 0.2% potato starch-Sigma 2004-). Results are shown in Table 5.

Table 5: Activity Spectrum of Hyicin M51

Strain	MIC (g/ml)
Staphylococcus aureus V557	1.56
Staphylococcus aureus Newbould	<0.39
Streptococcus uberis	0.78
Streptococcus faecalis	3.13
E.coli >100	
Pasteurella haemolytica	50
Listeria innocua	12.5
Bacillus cereus	25
Micrococcus luteus	<0.39
Leuconostoc mesenteroides	<0.39

I) **Experiments for evaluating the potential of Hyicin M51 or its pharmaceutically acceptable acid addition salts in subclinical bovine mastitis**

A dairy herd is maintained to supply candidate cows for enrollment in trials. Cows are bought from local cattle dealers. These cows should have a minimal milk production of 15 l/day. Two milk samples are taken one day apart from all quarters for bacteriological examination. No cows from which mastitis pathogens can be recovered are entered into the tests. Non-infected cows or goats are selected based on the lack of recovery of Staphylococcus aureus from two milk samples taken one day apart from all quarters. Then, 3 quarters are inoculated via the intramammary route using a suspension of Staphylococcus aureus. The 4th quarter serves as a control. In goats 1 of 2 udder halves are inoculated. During the two to four weeks after inoculation, milk samples are taken from all quarters at least 3 times to see whether Staphylococcus aureus can be recovered, and therefore whether the quarter has become infected. A quarter is defined as being infected, if Staphylococcus aureus is recovered from at least two of the milk samples. Treatments are assigned randomly to infected quarters and injected via the intramammary route. Milk samples are taken daily until a minimum of 14 days after the last treatment. A quarter is defined as being cured if milk samples from that quarter become negative (i.e., no Staphylococcus aureus is recovered) within 7 days after the last treatment and stay negative throughout the sampling period.

This experimental model is used to have a fixed framework on which to evaluate treatments. To work with Staphylococcus aureus has the advantage that it is the most difficult Gram positive bacterium to treat and one of the most important bacteria causing bovine mastitis. Therefore, the cure rates are expected to be lower than for other Gram positive bacteria, such as Streptococci and coagulase-negative Staphylococci.

This model has proved satisfactory since the infection rate in quarters averages 60%. In addition, the infection is mild as required for subclinical mastitis.

To optimize work load in relation to man power, 8 to 16 cows or goats are enrolled in each trial. Trials normally last 5 weeks.

Table 6: Efficacy of a hyicin M51 treatment in cows

Treatment Group	3 x 0 mg hyicin M51 each 12 hours	3 x 2 mg hyicin M51 each 12 hours	3 x 300 mg ampiclox each 12 hours
days after start of treatment	% of quaters positive (n=45)	% of quaters positive (n=?)	% of quaters positive (n=68)
0	97.7	85	100
1	75.6	4	19
2	77.8	7	1
3	80.0	15	7
4	73.3	30	6
5	77.8	67	24
7	75.6	70	29
8	75.6	70	34
9	71.1	63	35
10	75.6	67	35
11	71.1	63	43
12	64.4	67	41
14	68.9	63	43
15	80.0	67	43
16	80.0	70	

ANALYTICAL METHODS

1) Data Management and Analysis

A data entry/management system, created using the well known statistical analysis computer program SAS, is employed.

2) Definition of Cure Rate

Frequently, quarters are treated as independent statistical units and cure rates are based on results from individual quarters. This is not correct, since in the same cow quarters do not behave independently. In the literature, the correlation between quarters in a cow is estimated to be 0.25. From our data, we estimate that the correlation in this disease model is between 0.15 and 0.35.

We use a method to compute a cow cure rate for given treatment accounting for the total number of quarters infected and subsequently treated in a cow (1, 2 or 3), the number of quarters that cured in that cow, and the correlation between quarters. One consequence of using such a method is that more weight is given to a cow for which three out of three quarters cured compared to three cows for which one out of one quarter cured. This result coincides with the observation that the more quarters are infected, the less they will cure. This problem does not arise in goats, because we infect one half only of each goat's udder.

3) Other Factors Influencing Cure

Other factors are known or suspected to influence cure. This is why natural cure can occur in up to 20 % of cases. Some of the factors are "cow" factors (e.g.: breed or lactation number), others are "quarter" factors (e.g.: position of the quarter on the udder).

We consider and if necessary adjust for the following cowfactors in the analysis: breed, age, lactation number, lactation stage, daily milk production prior to treatment and number of times used for trials. Appropriate adjustments are also made for goats.

Quarter factors that must be considered are the following: number of bacteria present before treatment, number of bacteria present at the time of treatment, SCC at treatment, position (front or rear), infection history, and treatment history.

No statistical analysis exists that allows us to examine cow and quarter factors simultaneously. Therefore, we transform some quarter factors into cow factors. We define a median level of infection for a cow based on levels of infection in all its infected quarters, and we define a median SCC for a cow based on SCC of all its infected quarters.

We are aware that such pooling of data might prevent us from detecting the effect of quarter factors but, at present, we have no alternative. However, we do not think that the final interpretation of results will be compromised, since these factors are generally balanced between treatment groups. For instance, rear quarters cure less than fore quarters (30 % vs 45 %) but the proportion of rear quarters in all treatment groups varied between 30 and 40 %.

4) **Comparison of efficacy between treatments**

To compare the efficacy between treatments adjusting for the above mentioned factors, the odds ratio is calculated. These adjusted odds ratios compare the chance of curing between two treatments, all other confounding factors being held constant.
For instance if the odds ratio between treatments A and B is 3 it means that cows treated with A are three times more likely to cure than those treated with B. A 95 % confidence interval for the odds ratio is computed. Treatments are statistically different when the 95 % confidence interval does not include 1.

5) **Teat dipping and mastitis prophylaxis**

Teat dipping is one of the most frequently used and most effective measures taken to prevent mastitis in dairy cows. This widely practiced procedure involves dipping the 4 teat of every cow, after every milking, in an antibacterial solution. This procedure leaves a coating of antibacterial substances on the teat surface and a droplet of this substance collects at the dependent part of the teat over the teat orifice. After milking a droplet of milk often covers the teat orifice and is a good medium for bacterial growth. From this position bacteria can readily penetrate the streak canal. Teat dipping provides protection against these possibilities.

In some herds,teats are dipped before milking to kill off any pathogens resident on the teat surface before the milking machine is applied. To be useful as a pre-milking teat dip, agents need to be non-toxic and non-disruptive to cheese and yoghurt manufacture.

Products currently used for teat dipping contain a variety of different active ingredients such as iodophors, chlorhexidiene and the lanthionine containing peptide, nisin.
hyicin M51 and its pharmaceutically acceptable acid additional salts are very suitable for use in teat dips because of their rapid killing effect and preferential activity against major mastitis pathogens. Rapid killing gives a special application as an agent for pre-dipping.

6) **Teat dip protocol**

To evaluate the potential of hyicin M51 and its pharmaceutically acceptable acid addition salts as prophylactic agents in bovine mastitis, the following experimental protocol can be used.

Experimental animals: 50 cattle are recruited according to the protocol already described for trials of hyicin M51 in therapy of subclinical staphylococcal mastitis. This trial then proceeds according to protocol recognised by the US National Mastitis Council (NMC) (Hogan et al. 1990) for testing efficacy of teat germicides during experimental exposure.

Throughout the trial period all teats are dipped in a challenge bacterial suspension containing $15x10^7$Streptococcus aureus and $5x10^7$ Streptococcus agalactiae after the evening milking on week days. Challenge suspensions are prepared daily from stock solutions that are made up weekly. Aliquots of challenge solution are plated each day to determine the real bacterial concentration present at the time of challenge.

After each milking the left fore and right hind quarters are dipped in the test preparation of germicide, the other two teats remain as negative controls and are not dipped. Routine foremilk samples of all quarters are taken twice per week throughout the trial, for bacteriological examination. Any quarter, from which either challenge organism is isolated, is resampled within 48 hours of the original sample. If the same organism is isolated then the quarter is considered infected. Similarly an episode of clinical mastitis caused by either challenge organism is considered as a new intramammary infection (IMI).

At the end of the trial period the number of new intramammary infection between treatment groups is compared.

7) Hyicin M51 in Cheese and Yoghurt

To evaluate the potential effects of hyicin M51 or its pharmaceutically acceptable acid addition salts on strains of bacteria used in cheese and yoghurt manufacture the following experiments can be performed.

7.1 Evaluation of kinetics of reaction of Delvotest P in milk from quarters treated with Hyicin M51 or its pharmaceutically acceptable acid addition salts

Background information: Delvotest P is a commercially available, widely used, kit for detection of antimicrobial agents in milk. It relies on inhibition of the growth of Bacillus stearothermophilus. The indicator bacteria are suspended in an agar plug in a plastic test tube together with a pH indicator. Growth of bacteria results in acid production and colour

change. In the presence of inhibiting substances the bacteria do not grow and there is no colour change. Bacillus stearothermophilus is a good representative of bacteria used in cheese and yoghurt manufacture and hence it is widely used as the indicator strain in antibiotic residue detection.

Experimental method: This experiment is performed at the same time and on the same animals, as the efficacy trials. During the period following administration of various treatments samples are taken at morning and evening milkings. These samples are evaluated using Delvotest P as directed in the manufacturers instructions. This twice daily sampling goes on until all treated quarters show a negative response. In this way the duration of elimination of the antibacterial agent can be established.

7.2 Evaluation of effects of various concentrations of Hyicin M51 or its pharmaceutically acceptable acid addition salts on acidification of milk by cheese starter bacteria

In cheese and yoghurt manufacture different strains of bacteria are fermented in milk and one of the most important and immediate changes resulting from this fermentation is acid production. This acid is responsible for the control of secondary bacterial growth that could cause spoilage of the product or worse, illness in the consumer. Further fermentation of the lactic acid producing bacterial population produces changes in the cheese that vary with the strains of the bacteria used and the manufacturing methods. These factors are responsible for the physical characteristics and the flavour of different cheeses.

Acidification is the first and most important step in the manufacturing processes when milk is fermented. If acidification proceeds normally then it is very likely that all other steps will be normal. Therefore one can investigate the ability of different concentrations of hyicin M51 or its pharmaceutically acceptable acid addition salts on the kinetics of acidification of milk by different bacterial strains used in these manufacturing processes.

Protocol: Experiments are routinely performed using one of the following three strains of bacteria:

Streptococcus thermophilus
Lactobacillus bulgaricus
Lactobacillus lactis - diacetylactis

Preparation of stock cultures: Lyophylised cultures as used in commercial conditions are obtained from Rudolf Wittwer, CH-5002 Rombach, Aarau, Switzerland. These cultures are reconstituted according to the manufacturers instructions and reactivated by passage three times in sterile skimmed milk. Each passage is incubated for 16 - 18 hours at the optimum temperature for each bacterial strain.
The culture obtained after the third passage is diluted to 2% with whole sterile (UHT) milk, split into aliquots in 10ml tubes and frozen at -20° C for use in experiments.

Preparation of experimental culture: The stock cultures prepared above are thawed at 40° C, then incubated overnight at 37° C, and used to inoculate test samples at a concentration of 2% in whole sterile (UHT) milk.

Preparation of test samples: The antimicrobial agent under test (hyicin M51 or one of its pharmaceutically acceptable acid addition salts) is diluted in sterile (UHT) whole milk over the required dilution range. These samples are then inoculated with 2% by volume of the experimental culture of the strain of bacteria being tested. The samples are then mixed and incubated at optimal temperature for the bacterial strain under evaluation. The pH is measured each two hours for a period of eight hours and then at 18 hours after inoculation. The rate of acidification of milk containing different concentrations of active ingredients can then be compared to the rate without any active ingredient.

The invention now being fully described it will be apparent to one of ordinary skill in the art, that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

DEPOSIT

Strain	Accession Number
Staphylococcus hyicus 664	DSM 9547

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: Antimicrobial Compound

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGGAAAAAA TTCTTGATTT AGATGTACAA GTAAAGCGCA AAAAGGAATC AAATGATTCA 60

TCTGGAGATG AACGTATTAC AAGTTTCTCA TTGTGTACTC CTGGTTGCGC TAAAACTGGT 120

AGCTTCAATA GTTATTGTTG TTAA 144

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTTTTTGATC TTGACGTTAA AGT 23

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAATTTAAT TCTTTTGAAT T 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Glu Lys Ile Leu Asp Leu Asp Val Gln Val Lys Ala Gln Lys Glu
1               5                  10                  15

Ser Asn Asp Ser Ser Gly Asp Glu Arg Ile Thr Ser Phe Ser Leu Cys
            20                  25                  30

Thr Pro Gly Cys Ala Lys Thr Gly Ser Phe Asn Ser Tyr Cys Cys
        35                  40                  45
```

What we claim is:

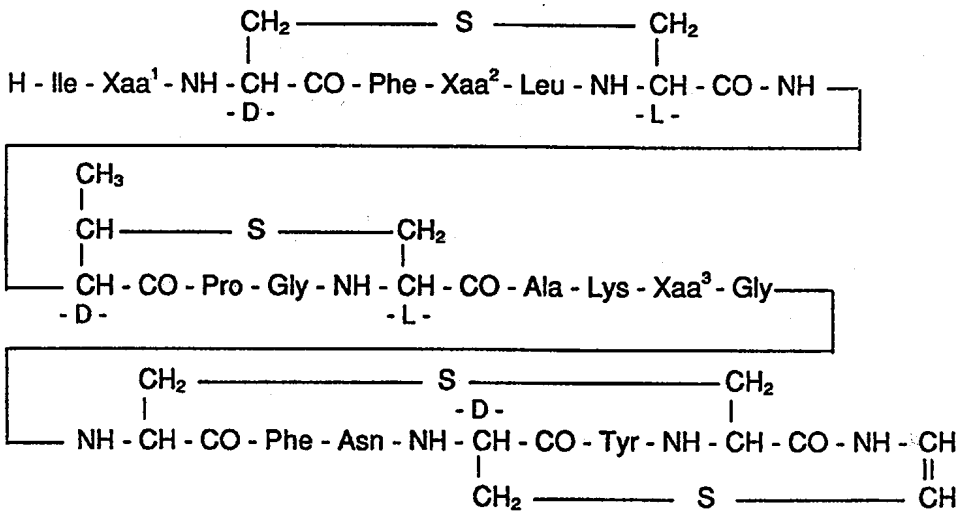
1. A lanthionine-containing antimicrobial peptide obtainable from the culture medium of the bacterial strain Staphylococcus hyicus strain 664, or a pharmaceutically acceptable acid addition salt thereof.

2. The peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof having the molecular weight of about 2119 ± 2 Dalton.

3. The peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof, which upon cleavage by trypsin yields two peptides of about 1256 ± 2 Dalton and about 881 ± 2 Dalton, respectively.

4. The peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof having an unusual amino acid at position 2 of the peptide chain.

5. The peptide according to claim 1 having the structure



wherein Xaa^1, Xaa^2, and Xaa^3 can be any amino acid with the proviso, that the molecular wheight of the complete peptide is 2119 ± 2 Dalton.

6. The peptide according to claim 5, wherein Xaa^1 and Xaa^3 are 2,3-Didehydrobutyrin (Dhb), and Xaa^2 is 2,3-Didehydroalanine (Dha).

7. The peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof showing antimicrobial activity against a bacterial strain selected from the group consisting of Staphylococcus aureus V557, Staphylococcus aureus Newbould 305, Streptococcus uberis, Streptococcus faecalis, Pasteurella haemolytica, Listeria innocua, Bacillus cereus, Micrococcus luteus, and Leuconostoc mesenteroides.

8. The peptide according to claim 7 or a pharmaceutically acceptable acid addition salt thereof having a Minimal Inhibitory Concentration (MIC) in milk of 0.01 g/ml to 100 g/ml.

9. The native peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof.

10. The peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof, which is of synthetic or recombinant origin, or is a mutein thereof.

11. The peptide according to claim 1, which peptide is obtainable by
    (a) growing a culture of the bacterial strain Staphylococcus hyicus strain 664 in a medium and for a time period appropriate to produce a peptide according to claim1,
    (b) purifying the peptide from the culture medium applying conventional techniques of protein purification, and
    (c) drying the active fractions.

12. The peptide according to claim 11, wherein the step of growing Staphylococcus hyicus strain 664 is done in BHI medium.

13. The peptide according to claim 11, wherein the step of purifying involves
    (a) heat-inactivation of the bacteria and adjusting the pH to 4.9 using 3M HCl;
    (b) adsorption of protein to XAD-7 Amberlite chromatographic column material and washing of the resin with water and 20mM Na-citrate pH4.0 containing up to 20% isopropanol;

(c) elution of the peptide from the XAD-7 Amberlite using 20mM Na-citrate pH4.0 containing 30% isopropanol;

(d) adsorption of protein to SP Sepharose Fast Flow chromatographic column material and washing of the resin with 0.01M HCl containing 30% methanol;

(e) elution of the peptide from SP Sepharose Fast Flow using 0.01M HCl containing 30% methanol and 0.3M NaCl;

(f) adsorption of protein to XAD-7 Amberlite chromatographic column material and washing of the resin with 0.01M HCl;

(g) elution of the peptide from the XAD-7 Amberlite using 0.01M HCl containing 50% isopropanol.

14. The peptide according to claim 11, wherein the step of drying involves lyophilisation of the active fractions.

15. An antimicrobial composition comprising in addition to a suitable carrier an antimicrobially effective amount of a peptide or a pharmaceutically acceptable acid addition salt thereof according to claim 1.

16. A pharmaceutical composition comprising in addition to a suitable carrier a pharmaceutically effective amount of a peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof.

17. A pharmaceutical composition according to claim 16 for the treatment or prevention of mastitis in mammals.

18. A nucleic acid molecule comprising a nucleotide sequence encoding the animicrobial peptide according to claims 1-14.

19. A DNA molecule according to claim 18.

20. A RNA molecule according to claim 18.

21. A DNA molecule according to claim 19 comprising the nucleotide sequence

ATG GAA AAA ATT CTT GAT TTA GAT GTA CAA GTA AAG CGC AAA AAG GAA
TCA AAT GAT TCA TCT GGA GAT GAA CGT ATT ACA AGT TTC TCA TTG TGT
ACT CCT GGT TGC GCT AAA ACT GGT AGC TTC AAT AGT TAT TGT TGT TAA

22. A RNA molecule according to claim 20 comprising the nucleotide sequence

AUG GAA AAA AUU CUU GAU UUA GAU GUA CAA GUA AAG CGC AAA AAG GAA
UCA AAU GAU UCA UCU GGA GAU GAA CGU AUU ACA AGU UUC UCA UUG UGU
ACU CCU GGU UGC GCU AAA ACU GGU AGC UUC AAU AGU UAU UGU UGU UAA

23. A method of treating or preventing mastitis in a mammal comprising administering to said mammal a pharamaceutically effective amount of a peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof.

24. The method according to claim 23 wherein the peptide is of native, recombinant or synthetic origin, or is a mutein thereof exhibiting activity against mastitis in a mammal.

25. The method according to claim 23 or 24, wherein said mammal is a cow, goat or ewe.

26. The method according to claim 23, wherein the peptide is administered via intramammary injection or by dipping the teat.

27. The method according to claim 23, wherein the peptide is administered via intramammary injection during the prepartum period.

28. The method according to claim 23, wherein the peptide is administered via intramammary injection during the postpartum period.

29. A method of preventing mastitis in a cow comprising intramammary injection of a pharmaceutically effective amount of a peptide according to claim 1 prior to infection.

30. A method of treating mastitis in a cow comprising intramammary injection of a pharmaceutically effective amount of a peptide according to claim 1 after onset of infection.

31. A method of preparing a peptide according to claim 1 comprising
    (a) growing a culture of the bacterial strain Staphylococcus hyicus strain 664 in a medium and for a time period appropriate to produce a peptide according to claim1,
    (b) purifying the peptide from the culture medium applying conventional techniques of protein purification, and
    (c) drying the active fractions.

32. A method according to claim 26, wherein the step of purifying involves consecutive steps of column chromatography.

33. A method of preparing a composition according to claim 16 or 17 comprising mixing a compound according to claim1 with a suitable carrier.

34. A method for producing a peptide according to claim 1 or a pharmaceutically acceptable acid addition salt thereof which is to be used for treating or preventing mastitis in a mammal.

35. A method of producing a nucleic acid molecule according to claims 18 to 22 comprising
    (a) construction of a genomic library of Staphylococcus hyicus strain 664 in a microorganism;
    (b) screening of said libary for sequences homologous to the structural gene of gallidermin;
    (c) identification of a clone comprising the structural gene of hyicin M51; and
    (d) purification of the nucleic acid molecule from the clone identified.

36. A method according to claim 35, wherein the library is constructed in E.coli.

37. Use of a peptide according to claim 1 to treat or prevent mastitis in a mammal.

38. Use of a composition according to claim 16 or 17 to treat or prevent mastitis in a mammal.

39. Use of a peptide according to claim 1 or 2 or a pharmaceutically acceptable acid addition salt thereof to prepare a drug for treating or preventing mastitis in a mammal.

40. Use according to claim 39, wherein the drug is a pharmaceutical composition for teat dipping.

41. Use of a nucleic acid molecule according to claim 18 to produce a peptide according to claim 1.

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/EP 95/01587

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/31 A61K38/16 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 181 578 (DR KARL THOMAE GMBH) 21 May 1986 see the whole document ---	1,9,10, 15-20, 32-34, 39-41
X	EP,A,0 342 486 (DR KARL THOMAE GMBH) 23 November 1989 see the whole document --- -/--	1,9,10, 15-20, 32-34, 39-41

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 1995	23.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/01587

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF DAIRY SCIENCE, vol.72, no.12, December 1989, CHAMPAIGN, ILLINOIS US pages 3342 - 3345 J R BROADBENT ET AL. 'Nisin inhibits several Gram-positive, mastitis causing pathogens' see the whole document ---	23-30, 37-40
X	NATURE., vol.333, 19 May 1988, LONDON GB pages 276 - 278 N SCHNELL ET AL. 'Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-ringd' see the whole document -----	1,9,10, 15-20, 32-34, 39-41

1

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/01587

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0181578	21-05-86	DE-A-	3440423	07-05-86
		DE-A-	3523478	08-01-87
		AU-B-	596082	26-04-90
		AU-A-	4941385	15-05-86
		CA-A-	1277617	11-12-90
		DE-A-	3583987	10-10-91
		HK-A-	63795	05-05-95
		JP-A-	6113826	26-04-94
		JP-B-	7032704	12-04-95
		JP-B-	6070078	07-09-94
		JP-A-	61158997	18-07-86
EP-A-0342486	23-11-89	ES-T-	2042868	16-12-93
		IE-B-	61615	16-11-94
		JP-A-	2117696	02-05-90
		PT-B-	90592	31-01-95
		US-A-	5231013	27-07-93

INTERNATIONAL SEARCH REPORT

:rnational application No.
PCT/EP 95/ 01587

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 23-30 and 37-38 refer to a method of treatment of the human/animal body the search was carried out and based on the alleged effect of the products.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [---] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.